Endothelial Progenitor Cells

Preserved Function of Late-Outgrowth Endothelial Cells in Medically Treated Hypertensive Patients Under Well-Controlled Conditions

Zhi Chen, Sandra M.S. Herrmann, Xiangyang Zhu, Kyra L. Jordan, Monika L. Gloviczki, Amir Lerman, Stephen C. Textor, Lilach O. Lerman

Abstract—Endothelial progenitor cells (EPCs) participate in renal repair, but their number and function may be impaired by exposure to cardiovascular risk factors. The number of circulating EPCs is decreased in essential and renovascular hypertensive patients, but the effects of hypertension on EPC function are incompletely understood. We hypothesized that EPC function was preserved under well-controlled conditions in treated hypertensive patients. Patients with atherosclerotic renal artery stenosis (ARAS; n=22) or essential hypertension (n=24) were studied during controlled sodium intake and antihypertensive regimen. Late-outgrowth EPCs were isolated from the inferior vena cava (IVC) and renal vein blood of ARAS and essential hypertension patients and a peripheral vein of matched normotensive controls (n=18). The angiogenic function of EPCs was assessed in vitro, and multidetector computed tomography was used to measure single-kidney hemodynamics and function in ARAS and essential hypertension patients. Inflammatory biomarkers and EPC homing signal levels and renal release were calculated. Inferior vena cava and renal vein–obtained EPC function were similar in ARAS and essential hypertension patients and comparable to that in normal controls (tube length, 171.86±16.846, 191.09±14.222, 174.925±19.774 μm, respectively). Function of renal vein–obtained EPCs directly correlated with stenotic kidney glomerular filtration rate, EPC homing factors, and anti-inflammatory mediator levels in ARAS patients. Therefore, EPC function was relatively preserved in ARAS patients, although it directly correlated with renal function. Adequate EPC function supports the feasibility of using autologous EPCs as a therapeutic option in essential and renovascular hypertensive patients. Homing signals and inflammatory mediators may potentially regulate EPC angiogenic function. (Hypertension. 2014;64:808-814.) ● Online Data Supplement

Key Words: hypertension, essential □ hypertension, renovascular

Atherosclerotic renal artery stenosis (ARAS) is the major cause of secondary hypertension, which is characterized by reduced renal perfusion. Population studies revealed that ARAS constitutes an important risk factor for cardiovascular morbidity and mortality compared with essential hypertension (EH).1 We have also previously shown that ARAS exacerbates renal inflammation, oxidative stress, and renal release of cytokines in both experimental models2 and patients.3

A large body of evidence has shown that circulating bone marrow–derived endothelial progenitor cells (EPCs) contribute to endothelial repair in ischemic tissues.4 These cells have been described to include 2 subpopulations, based on the stage at which they appear in culture. Early-outgrowth endothelial cells appear after 4 to 7 days and show monocytc features, and late-outgrowth endothelial cells (LOECs) appear after 14 to 21 days in culture, possess salient angiogenic capabilities, and exhibit many features resembling bone marrow–derived circulating EPC.5 Furthermore, a combination of autologous early-outgrowth endothelial cells and LOECs expanded in vitro has been used to replenish injured kidneys and improve their function in chronic experimental ARAS.6–8

However, EPC function may be impaired by exposure to cardiovascular risk factors such as hypertension or diabetes mellitus,9,10 partly because of decreased nitric oxide availability or activation of the renal–angiotensin–aldosterone system (RAAS). Activation of angiotensin-II in ARAS is involved in atherogenesis, inflammation, oxidative stress, and endothelial damage11 and is particularly prominent within the kidney. Furthermore, we have previously shown that the number of systemic circulating CD34+/KDR+ EPCs is decreased in both ARAS and EH patients compared with healthy volunteers (HVs), yet is further decreased in the renal vein (RV) effluent of the post–stenotic ARAS kidneys (STKs) compared with EH kidneys. Pertinently, we have demonstrated...
that inflammatory cytokines released from the human STK recruited and sequestered circulating EPCs to participate in the reparative process. Nevertheless, EPC crossing the circulation of the STK might be functionally damaged because of the noxious microenvironment. Importantly, impaired LOEC function would imply impaired endogenous renal regeneration capacity in ARAS patients and argue against the use of LOEC to replenish the diminished population of EPCs available for renal repair.

However, it remains unknown whether LOEC function in ARAS patients is adequate and affords reparative activity. The current study was designed to test the hypothesis that angiogenic function of LOEC expanded from the systemic circulation and STK of ARAS patients is preserved compared with that in EH and HVs.

Methods

This study was approved by the Institutional Review Board of the Mayo Clinic. ARAS (n=22) or EH (n=24) patients were prospectively enrolled, and HVs (n=18) without cardiovascular risk factors were prospectively recruited through the Mayo Clinic Biobank. In all EH or ARAS patients, the antihypertensive regimen included RAAS blockade. During a 3-day inpatient protocol, STK renal blood flow and glomerular filtration rate (GFR) were measured using multidetector computed tomography. Blood samples were collected from the IVC and RV of ARAS and EH patients and from peripheral vein of HVs for measurements of inflammatory marker levels, EPC isolation, and subsequent evaluation of their proliferation, migration, and tube formation function in vitro. For detailed methodologies, see the online-only Data Supplement.

Results

Patients Characteristics

The characteristics of study subjects are summarized in the Table. Systolic blood pressure was higher in EH and ARAS patients compared with HVs. Antihypertensive medications were similar between EH and ARAS.

Kidney Function

Serum creatinine levels were elevated in ARAS compared with HVs (Table), but urine protein excretion was unaltered. Systemic plasma renin activity levels were elevated in both ARAS and EH compared with HVs. Single-kidney function in the 3 groups is presented in Table S1 in the online-only Data Supplement. Of the ARAS patients, 10 of 22 were found to have bilateral disease, in which case both kidneys were considered as STK. Single-kidney cortical perfusion was decreased in STK compared with both EH and nonstenotic, contralateral kidneys (CLKs), and in CLK compared with EH. Medullary perfusion was decreased in STK compared with EH, whereas STK renal blood flow and GFR were both reduced compared with EH and CLK. RV plasma renin activity was elevated in STK compared with EH.

LOEC Function In Vitro

Cultured LOEC had a cobblestone appearance (indicative of endothelial-like phenotype) and expressed CD34 and KDR.

Table. Characteristics of HVs, EH, and ARAS Patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HVs (n=18)</th>
<th>EH (n=24)</th>
<th>ARAS (n=22)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>69.1±1.8</td>
<td>69.8±1.5</td>
<td>70.2±1.5</td>
<td>0.88</td>
</tr>
<tr>
<td>Sex, men/women</td>
<td>11/7</td>
<td>15/9</td>
<td>12/10</td>
<td>0.85*</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.3±1.0</td>
<td>27.0±0.9</td>
<td>27.8±0.9</td>
<td>0.54</td>
</tr>
<tr>
<td>Degree of stenosis, %</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>74.2±0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Systolic pressure, mm Hg</td>
<td>120.4±2.8</td>
<td>132.5±2.4†</td>
<td>137.8±2.6†</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diastolic pressure, mm Hg</td>
<td>70.8±1.9</td>
<td>69.3±2.3</td>
<td>69.9±2.8</td>
<td>0.57</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>87.7±1.7</td>
<td>90.3±1.9</td>
<td>90.9±2.1</td>
<td>0.50</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>177.7±6.7</td>
<td>174.8±5.8</td>
<td>170.8±6.0</td>
<td>0.76</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>117.1±17.5</td>
<td>123.8±11.4</td>
<td>133.7±13.3</td>
<td>0.26</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>57.0±3.6</td>
<td>53.1±2.7</td>
<td>55.1±4.4</td>
<td>0.42</td>
</tr>
<tr>
<td>LDL, mg/dL</td>
<td>90.3±4.6</td>
<td>96.8±4.4</td>
<td>88.9±4.7</td>
<td>0.43</td>
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<tr>
<td>Serum creatinine, mg/dL</td>
<td>0.93±0.04</td>
<td>1.03±0.06</td>
<td>1.24±0.07†</td>
<td>0.005</td>
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<tr>
<td>Proteinuria, mg/24 h</td>
<td>86.9±7.4</td>
<td>90.5±13.2</td>
<td>76.6±11.0</td>
<td>0.24</td>
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<tr>
<td>Microalbuminuria, mg/24 h</td>
<td>0/0</td>
<td>33.0±5.9</td>
<td>29.1±8.1</td>
<td>0.68</td>
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<tr>
<td>Systemic PRA, ng/mL per hour</td>
<td>0.51±0.1</td>
<td>6.4±1.4†</td>
<td>8.9±1.8†</td>
<td>0.0002</td>
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<td>Calcium-channel blocker</td>
<td>0/0</td>
<td>9/37.5</td>
<td>8/36.4</td>
<td>0.84*</td>
</tr>
<tr>
<td>ACE-I</td>
<td>0/0</td>
<td>16/66.7</td>
<td>12/54.5</td>
<td>0.40*</td>
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<tr>
<td>ARBs</td>
<td>0/0</td>
<td>12/50.0</td>
<td>15/68.2</td>
<td>0.21*</td>
</tr>
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<td>α-Blockers</td>
<td>0/0</td>
<td>2/8.3</td>
<td>1/4.5</td>
<td>0.60*</td>
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<tr>
<td>Statins</td>
<td>0/0</td>
<td>13/54.2</td>
<td>14/63.6</td>
<td>0.51*</td>
</tr>
</tbody>
</table>

Data are mean±SEM or n (%). ACE-I indicates angiotensin-converting enzyme inhibitor; ARAS, atherosclerotic renal artery stenosis; ARB, angiotensin receptor blocker; BMI, body mass index; EH, essential hypertension; HDL, high-density lipoprotein; HV, healthy volunteer; LDL, low-density lipoprotein; MAP, mean arterial pressure; and PRA, plasma renin activity.

*Fisher exact test between EH and ARAS.
†P<0.05 vs HVs.
Migration activity, proliferation, and tube formation of LOEC obtained from either collection site was all similar among the 3 groups (Figure 1A–1F).

The STK and CLK–RV–LOEC migration and proliferation, respectively, directly correlated with the corresponding single-kidney GFR in ARAS and their migration with CLK cortical perfusion. The STK–RV–LOEC migration tended to correlate with STK cortical perfusion (Figure 2A–2D). The presence of bilateral disease did not significantly affect these relationships (Figure S2).

Conversely, the EH–RV–LOEC migration correlated inversely with the corresponding cortical perfusion and GFR (Figure 2E and 2F). In addition, EH–RV–LOEC proliferation inversely correlated with systolic blood pressure, and tube formation with diastolic and mean arterial pressure (Figure 2G–2I).

We have also observed significant correlations between LOEC functions and homing and inflammatory mediator levels in the corresponding sites. The HV and CLK–RV–LOEC tube formation directly correlated with granulocyte–macrophage colony-stimulating factor (GM-CSF; Figure 2J and 2K). Similarly, both systemic and RV–LOEC migration directly correlated with IVC and RV level of GM-CSF in EH (Figure 2L and 2M), and the STK–RV–LOEC migration directly correlated with the renal release of G-CSF and interleukin-10 (Figure 2N and 2R). The ARAS–IVC–LOEC migration also directly correlated with stromal-derived factor-1 and that of STK and CLK–RV with monocyte chemoattractant protein-1 (Figure 2O–2Q).

Stratification based on statin treatment did not reveal significant differences between LOEC obtained from treated and untreated patients (Figure S3).

No other statistically significant correlations were detected.

**Figure 1.** Extrarenal (peripheral or inferior vena cava) and renal vein levels of late-outgrowth endothelial cell (LOEC) migration, proliferation, and tube formation capacity in healthy controls, essential hypertensive (EH) patients, or the stenotic kidneys (STKs) and contralateral kidneys (CLKs) of patients with atherosclerotic renal artery stenosis (ARAS). LOEC function was similar among the groups regardless of whether they were expanded from the extrarenal (A–C) or renal (D–F) veins. Representative tube formation images (bottom).
Figure 2. Correlation of late-outgrowth endothelial cell (LOEC) function with renal hemodynamics, glomerular filtration rate (GFR), inflammatory markers or homing signals in healthy volunteers (HVs), atherosclerotic renal artery stenosis (ARAS), and essential hypertensive (EH) patients. A to D, In ARAS, renal vein (RV)–LOEC migration and proliferation directly correlated with single-kidney GFR in stenotic (STK) and contralateral (CLK) kidneys and migration with CLK cortical perfusion. The STK–RV–LOEC migration tended to correlate with STK cortical perfusion. E and F, Conversely, EH–RV–LOEC migration inversely correlated with single-kidney cortical perfusion and GFR. G to I, EH–RV–LOEC proliferation correlated inversely with systolic blood pressure (SBP), and tube formation with diastolic blood pressure (DBP) and mean arterial pressure (MAP). J and K, Tube formation of CLK–RV and HV systemic LOEC directly correlated with the level of granulocyte–macrophage colony-stimulating factor (GM-CSF). L and M, Similarly, both systemic and RV–LOEC migration directly correlated with the level of GM-CSF in EH patients. N to R, In ARAS, the STK–RV–LOEC migration directly correlated with the renal release of G-CSF and interleukin-10 (IL-10). Also, the systemic LOEC migration directly correlated with stromal-derived factor-1 (SDF-1) level and RV–LOEC migration with monocyte chemoattractant protein-1 (MCP-1). S and T, IV–LOEC migration inversely correlated with tumor necrosis factor-α (TNF-α) level in ARAS and tube formation of EH–RV–LOEC with interferon-γ (IFN-γ).
Discussion
In the present study, we demonstrate that LOEC angiogenic activity is preserved in treated renovascular and EH patients compared with normotensive controls. Moreover, LOEC expanded from either the RV or the systemic circulation showed comparable migratory, proliferative, and tube-forming capacities to those of HV systemic LOEC. These findings, therefore, support the use of ARAS and EH–LOEC for reparative purposes during renal injury.

We have previously found that the numbers of EPC were diminished in the systemic circulation of patients with EH or renovascular hypertension and further reduced in the STK compared with the EH RV. However, after 12 weeks of experimental renovascular hypertension in swine, LOECs showed unaltered proliferation and tube formation compared with normotensive pigs. Similarly, the present study shows that in patients with prolonged but medically treated hypertension under controlled conditions, LOECs expanded from RV and systemic blood showed angiogenic capacity comparable with HVs.

Previous studies have shown that hypertension attenuates the numbers of circulating EPCs and colony forming units via increased tissue angiotensin-II. Angiotensin-II induces reactive oxygen species production, which reduces EPC levels, impairs their function, and accelerates EPC senescence. Inhibitors of RAAS, such as angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, may have beneficial effects on the number and function of EPC in cardiovascular disease, including hypertension and atherosclerosis. For example, Losartan decreased the expression of reactive oxygen species–forming enzymes in the rat aorta, heart and kidney, resulting in increased EPC number and function. Similarly, angiotensin receptor blockers improved the impaired EPC angiogenic capacity in EH patients. In the present study, the angiogenic capacity of LOEC obtained from the RV or systemic blood in our hypertensive patients was not impaired compared with healthy controls, possibly because they were all treated with RAAS inhibitors. Additionally, the inverse correlation between LOEC function and blood pressure in EH patients suggested reduction of blood pressure as a potential mechanism of anti-hypertensive drugs in enhancing LOEC angiogenic capacity.

LOEC migration, proliferation, and tube formation capacity in ARAS patients were also maintained. ARAS is characterized by renal hyperperfusion, activation of angiotensin-II, increased reactive oxygen species generation, and microvascular loss, as well as vascular wall and microvascular remodeling. Coexistence of atherosclerosis and persistent hyperperfusion in ARAS kidneys magnifies oxidative stress and inflammation. As observed in other chronic kidney diseases, RAAS inhibitors attenuate oxidative stress and inflammation, slow the progression of microvascular remodeling, and restore EPC levels and function. Collectively, these effects may account for the preserved LOEC function in ARAS patients. Contrarily, this strategy was less effective in sustaining circulating EPC numbers that decline in such patients, suggesting a different regulatory mechanism of EPC number and function.

Notably, most of our hypertensive patients were treated with statins and calcium channel blockers, which might also attenuate EPC apoptosis and increase EPC mobilization and differentiation. Thus, their pleiotropic effects may have also improved LOEC function. However, this similar relationship between LOEC and renal function in patients stratified by statin treatment argues against its effect on this relationship. Effects of other drugs will need to be tested in future studies designed for this purpose.

The type of EPC may also determine their functionality. Giannotti et al have shown that early-outgrowth endothelial cells obtained from patients with untreated hypertension and hypertension and expanded for 7 days have impaired function. We studied the function of LOEC obtained from patients treated with antihypertensive drugs under dietary and therapeutically controlled conditions, and with only mildly elevated systolic pressures. Hence, collectively, these results suggest that antihypertensive treatment may improve angiogenic function of circulating progenitor cells.

Nevertheless, despite medical treatment, we observed a significant relationship between LOEC function and single-kidney hemodynamics assessed using multidetector computed tomography. The migration capacity of only RV–LOEC correlated with STK GFR and tended to correlate with cortical perfusion. Furthermore, we found direct correlations between the migratory activity of RV–LOEC and CLK cortical perfusion, and between their proliferation and CLK GFR. These observations suggest that in the ARAS STK and CLK, hemodynamics and function are determinants of the angiogenic potency of reparative cells that transit within their circulation, possibly by virtue of the intrarenal inflammatory and pro-oxidant microenvironment. Interestingly, we conversely observed an inverse correlation between IVC or RV–LOEC migration and both cortical perfusion and GFR in EH patients. Possibly in the EH kidney, hyperfiltration produces a noxious microenvironment that impairs EPC function akin to that caused by ischemia in the STK. Yet, these effects on EPC function are likely subtle, given that overall EPC function in ARAS and EH patients was not different than that in HVs.

In response to renal injury in ARAS, the STK releases inflammatory cytokines and specific injury-homing signals to recruit and retain circulating EPCs to stimulate its reparative process. We previously observed an inverse correlation between the number of EPCs and RV level of tumor necrosis factor-α and interferon-γ in EH and ARAS patients. The current study extends our previous observations and demonstrates a significant inverse relationship between LOEC function and tumor necrosis factor-α or interferon-γ in ARAS and EH patients. Conversely, the anti-inflammatory cytokine interleukin-10 showed a direct correlation with LOEC migration. Therefore, inflammatory cytokines may play a role in regulating LOEC angiogenic function. Furthermore, levels of GM-CSF, G-CSF, and stromal-derived factor-1 correlated with LOEC function in all groups. These homing factors are all involved in the recruitment and mobilization of circulating progenitor cells from the bone marrow, partly by bolstering their function. These molecules and their cognate receptor expressed on progenitor cells mediate the homing
and engraftment process. Indeed, CXCR4 was expressed on our patients’ EPC. Thus, homing signals and inflammatory cytokines may regulate LOEC angiogenic function. Notably, RV level of monocyte chemoattractant protein-1 correlated directly with STK and CLK RV–LOEC function. Indeed, monocyte chemoattractant protein-1 has been shown to induce stem cell migration and recruitment, and the expression of its CCR2 receptor on EPC in this study poses this chemokine as a homing and retention factor in human ARAS.

Our study is limited by a relatively small sample size and cross-sectional nature. We also excluded patients with diabetes mellitus or serum creatinine levels >1.7 mg/dL, because of application of iodinated contrast for multidetector computed tomographic scanning. Therefore, changes of EPC function in these patients should be determined in future studies. The cause-and-effect relationships between renal hemodynamics, GFR, inflammatory markers, and progenitor cell function, and the effects of antihypertensive regimen on the relationship between LOEC and renal function also merit further investigation. In addition, the mechanism by which inflammatory mediators modulate LOEC function and changes in early-outgrowth endothelial cell function in our patient groups all await further studies.

**Perspectives**

Our results demonstrate preserved function of systemic and RV–LOEC in medically treated renovascular and EH patients. The correlation between LOEC function and renal function may imply that the ambient microenvironment exerts small but potentially meaningful effects on the function of EPCs that transit across the renal circulation. Moreover, the correlation between LOEC function and inflammatory markers and homing signals in both ARAS and EH patients suggests that inflammation may regulate EPC function. Identification of these effects may allow refining novel therapeutic options for patients with ARAS. Therefore, in medically treated patients with mildly elevated systolic blood pressure under well-controlled conditions, LOEC function is relatively maintained and may permit their utilization for autologous delivery for potential reparative applications in case of imminent renal injury.

**Sources of Funding**

This study was partly supported by National Institutes of Health grants DK73608, HL92954, AG17150, HL12156, DK100081, C06-RR018898, and the Mayo Clinic Center for Regenerative Medicine.

**Disclosures**

None.

**References**


### Novelty and Significance

**What Is New?**

- Our study demonstrates preserved endothelial progenitor cell (EPC) angiogenic function in patients with atherosclerotic renal artery stenosis compared with essential hypertensive patients and healthy volunteers. Yet, EPC function correlated with renal vein levels of anti-inflammatory mediator and EPC homing factors.

**What Is Relevant?**

- The adequate angiogenic function underscores EPC as a viable therapeutic option in essential and renovascular hypertensive patients.

### Summary

The relatively preserved EPC function supports the feasibility of using autologous EPCs as a therapeutic tool in essential and renovascular hypertensive patients. Furthermore, homing signals and inflammatory mediators may modulate EPC angiogenic function.
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Hypertension. 2014;64:808-814; originally published online July 21, 2014;
doi: 10.1161/HYPERTENSIONAHA.114.03720

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Preserved function of late outgrowth endothelial cells in medically treated hypertensive patients under well-controlled conditions

Chen et al, EPC function in renovascular disease

Zhi Chen¹,³ MD; Sandra M.S.Herrmann¹ MD; Xiangyang Zhu¹ MD, PhD; Kyra L. Jordan¹; Monika L. Gloviczki¹ MD, PhD; Amir Lerman², MD; Stephen C. Textor¹ MD; Lilach O. Lerman¹,² MD, PhD.

Divisions of ¹Nephrology and Hypertension and ²Cardiovascular Diseases, Mayo Clinic, Rochester, MN 55905;³Division of Nephrology, First Hospital of Jilin University, Jilin, China.

Total Word Count: 3961

Word Count of Abstract: 238

Address for correspondence:

Lilach O. Lerman, MD, PhD
Division of Nephrology and Hypertension, Mayo Clinic
200 First Street SW, Rochester, MN 55905
Fax: 507-266-9316
Telephone: 507-293-0890
E-mail: lerman.lilach@mayo.edu
METHODS

The study protocol was approved by the Institutional Review Board of the Mayo Clinic, and informed written consent was obtained from all participants. We prospectively enrolled 46 hypertensive patients using criteria comparable to our previous studies\(^1\). ARAS patients were identified using criteria similar to those that governed enrollment in the Cardiovascular Outcomes for Renal Atherosclerotic Lesions (CORAL)\(^2\) trial. Severity of renal artery stenosis was based on imaging criteria, including renal artery ultrasound velocity acceleration (peak systolic velocity >200 cm/s), MR/CT angiography with significant stenosis >60%, and/or post-stenotic dilation. Exclusion criteria included serum creatinine >1.7 mg/dl, patients with clinical evidence of symptomatic coronary artery disease, renal transplant and active inflammatory disease.

Healthy volunteers (n=18) without cardiovascular risk factors were prospectively recruited through the Mayo Clinic Biobank.

After enrollment, patients with ARAS (n=22) or EH (n=24) were admitted to the clinical research unit for 3 days, where their dietary intake of 150 mEq of sodium was regulated, as described previously\(^1\). In all patients with EH or ARAS, the antihypertensive regimen included blockade of the renin-angiotensin system using angiotensin-converting enzyme inhibitors (ACE-I) or angiotensin receptor blockers (ARBs) at the standard recommended daily dose. Their previous therapy with statins was continued.

All enrolled subjects underwent a detailed physical examination and laboratory biochemical measurement by standard procedures, and clinical and laboratory data collected including blood pressure, serum creatinine, systemic plasma renin activity (PRA), low density lipoprotein, high density lipoprotein, total cholesterol, triglycerides, and 24h urinary protein levels.

During the 3-day inpatient protocol study, ARAS and EH patients were cannulated via the femoral vein with a 5F Cobra catheter (Cook, Inc. Bloomington, IN) for subsequent multidetector CT (MDCT) scanning, and for selective collection of inferior vena cava (IVC) and renal vein (RV) samples for EPC isolation. EPC were cultured from all samples, and their proliferation, migration, and tube formation function evaluated in vitro.

Renal hemodynamics measurement in vivo

MDCT study was performed using a dual-source 64-slice helical MDCT scanner (Somatom Sensation 64, Siemens Medical Solutions, Germany) to assess single-kidney volume, perfusion, renal blood flow (RBF) and glomerular filtration rate (GFR), as described before\(^1,3,4\). After a central venous injection of iopamidol-370 (0.5 ml/kg, up to a maximum of 40 ml) using a power injector, perfusion scans were acquired in the sequential module, composed of a total of 45 consecutive scans over about 3 minutes\(^1\). After a 15-minute rest, a kidney volume study was performed in the helical mode to obtain contiguous 5-mm-thick slices for measurement of cortical, medullary and total kidney volume.
Images were then reconstructed on CT console and analyzed using the software package ANALYZE® (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). MDCT analysis was undertaken by manually selecting regions of interest in the kidney on the cross-sectional images from the aorta, bilateral cortex and medulla. Tissue attenuation curves over time were fitted using curve-fitting algorithms to measure individual kidney-cortical and medullary perfusion and normalized GFR5, and cortical and medullary volumes were calculated using stereology. Single-kidney RBF, GFR and regional flows were then determined by multiplying renal perfusion or GFR (mL/min/tissue), by the respective kidney volume5, and GFR also corrected for hematocrit6. The degree of renal arterial stenosis was determined using CT angiography.

Progenitor cells
Blood collection and cell isolation

Blood samples (40 mL from each site) were taken in hypertensive patients from the IVC and right and left RV prior to MDCT scanning, and in healthy controls from a peripheral (antecubital) vein, and were transferred immediately for analysis. Mononuclear cells were isolated by density-gradient centrifugation method7,8 using Histopaque 1077(Sigma, St. Louis) at 1600 rpm for 25 minutes, and resuspended in EGM-2 Single quot medium (Lonza, Walkersville, MD, USA). Cells were plated on 6-well plates (Corning Inc, Corning, NY) coated with fibronectin (1µg/m², Sigma, St. Louis, MO). After a 2-day culture, non-adherent cells were removed and the media changed daily for 1 week, and every other day for the remaining time. The culture was maintained for 3-4 weeks. After 4-10 days of culture, the attached cell population was composed of EOEC that exhibit some characteristics of EPC, but little proliferative capacity7. After 3-4 weeks of culture, the LOEC population appeared, comprised of larger cells with mature endothelial cell culture characteristic that exhibit higher rates of proliferation. The LOECs were then assayed for cellular phenotype and function and examined microscopically.

Characterization of LOEC marker expression

Immuno-fluorescence was performed to characterize these human LOECs7. LOECs were incubated with primary antibodies against CD34 (R&D, MN, Cat# AF3890, NS0-derived rpCD34) and KDR (Santa Cruz, CA, Cat# sc-504, Clone: C-1158) for 1h at room temperature. FITC-goat anti-mouse or anti-rabbit IgG were incubated for 30 min at room temperature. Nuclei were stained with DAPI (Santa Cruz, CA). All sections were examined under a fluorescence microscope.

Evaluation of LOEC function in vitro

LOEC function was evaluated by several accepted tests9,10.

Migration

LOEC migratory function was tested using a QCM Haptotaxis cell migration kit (Millipore, Billerica, Massachusetts, USA), as we have shown before9. Briefly, LOECs (1
x 10^6 cells/ml) were placed in fibronectin-coated wells and incubated for 24h; nonadherent cells were removed, and migrated cells stained. Then the stained LOECs were solubilized with extraction buffer, and 100μl buffers were transferred to a 96-well plate, and the LOEC migration was measured in a plate reader at absorbance 562nm.

Proliferation

LOEC proliferation function was examined using MTS (Cell Titer 96 Non-Radioactive Cell Proliferation Assay, Promega, Madison, Wisconsin). LOECs were seeded at 3×10^3/well in 96-well flat-bottom plates in EGM-2 medium containing 2% FCS and allowed to adhere for 24h. Then, MTS solution was added at 20 μl/well, and after 4h of culture, the conversion of MTS to formazan was measured at 490 nm.

Matrigel tube formation

To assess the ability of LOECs to incorporate into and form vascular structures, a matrigel tube formation assay was performed. Matrigel (BD Biosciences, Bedford, MA) was spread onto 24-well and allowed to polymerize for 15 min at 37°C. LOECs were labeled with Dil (Molecular Probes) and mixed with human umbilical endothelial cells (HUVEC, PromoCell, Heidelberg, Germany). Dil-labeled LOECs (1×10^4) and HUVEC (4×10^4) were plated together and incubated at 37°C for 24h with EGM-2 culture medium. The lengths of tubes formed by HUVEC were measured using Meta-Morph® image analysis software (Meta Imaging Series 6.3.2, Allentown, PA).

Inflammatory biomarkers measurement

RV and systemic blood samples were collected and centrifuged, then the level of granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), stromal-derived factor (SDF)-1, monocyte chemoattractant protein (MCP)-1, interlukin (IL) -10 and tumor necrosis factor (TNF-α) were measured by Luminex (Millipore, cat No: MPXHCYTO-60K; MPXHCYP2-62K; HCVD-67AK; and HSCR-32K). Signals were read by the Bio-plex 200 systems (BIO-RAD).

Based on the assumption that the different level between RV and IVC reflects net release of cytokines within the affected kidney, we calculated a cytokine gradient (RV-IVC) and renal net release (gradient × RBF) for each blood sample.

Statistical analysis

Statistical analysis was performed using JMP version 9.0 (SAS Institute, Cary, NC). Continuous data were expressed as mean±SEM for normally distributed variables, and as median (range) for data that did not show a normal distribution. All STK were compiled. Multiple group comparisons were performed using 1-way ANOVA, followed by post-hoc Tukey test, or using Wilcoxon signed rank test. Regressions were calculated by the least-squares fit, and variables that did not follow a normal distribution were first log-transformed. To investigate the effect of drug treatment on the correlation between LOEC function and renal hemodynamics and function, we stratified EH and ARAS groups based on statin treatment. P-values ≤0.05 were considered statistically significant.
REFERENCES


Table S1. Single-Kidney function in essential hypertensive (EH) and atherosclerotic renal artery stenosis (ARAS) patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>EH (n=24)</th>
<th>ARAS</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>STK (n=32)</td>
</tr>
<tr>
<td>Total kidney volume (ml)</td>
<td>143.8±6.8</td>
<td>107.5±8.8</td>
<td>169.0±12.8</td>
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<td>Cortical volume (ml)</td>
<td>93.1±4.9</td>
<td>68.1±5.6</td>
<td>107.9±9.2</td>
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<tr>
<td>Medullary volume (ml)</td>
<td>50.7±2.2</td>
<td>39.4±3.4</td>
<td>61.1±5.7</td>
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<td>Renal tissue perfusion</td>
<td></td>
<td></td>
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<tr>
<td>Cortical perfusion (ml/min/cc)</td>
<td>3.7±0.2</td>
<td>2.5±0.1</td>
<td>3.0±0.1</td>
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<td>Medullary perfusion</td>
<td>1.3±0.1</td>
<td>0.9±0.1</td>
<td>1.1±0.1</td>
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<tr>
<td>Renal blood flow (ml/min)</td>
<td>417.7±36.5</td>
<td>225.6±22.4</td>
<td>380.3±40.8</td>
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<tr>
<td>Cortical flow (ml/min)</td>
<td>354.2±33.4</td>
<td>185.9±19.6</td>
<td>314.1±36.8</td>
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<tr>
<td>Medullary flow (ml/min)</td>
<td>63.5±4.1</td>
<td>39.6±4.2</td>
<td>66.2±9.2</td>
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<tr>
<td>Single-Kidney GFR (ml/min)</td>
<td>41.7±2.5</td>
<td>25.9±3.0</td>
<td>36.7±4.4</td>
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<tr>
<td>PRA RV (ng/ml/h)</td>
<td>8.3±1.7</td>
<td>16.7±2.8</td>
<td>9.9±2.6</td>
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</tbody>
</table>

Data are mean±SEM. STK: stenotic kidney; CLK: contralateral kidney; GFR: glomerular filtration rate; PRA: plasma renin activity; RV: EH or STK renal vein. *p<0.05 vs EH, †p<0.05 vs CLK.
Figure S1 (A-B) Representative immunofluorescence images for surface markers of LOEC. **A:** Expression of progenitor (CD34, green) marker. **B:** Expression of endothelial (KDR, red) marker. After 21 days, the positive expression of endothelial marker suggested that progenitor cells acquired endothelial phenotype. (C-D) Representative images of homing signal receptors expressed on progenitor cells obtained from hypertensive patients. Both the SDF-1 CXCR4 receptor (C) and MCP-1 receptor CCR2 (D) were expressed on LOEC.
Figure S2 Correlation of the late outgrowth endothelial cell (LOEC) function with renal hemodynamics, renal function or inflammatory and homing markers in patients with bilateral renal artery stenosis. (A-B) The migration activity of LOEC expanded from stenotic kidneys (STK) tend to directly correlated with STK glomerular filtration rate (GFR), and the tube formation of STK RV-obtained LOEC directly correlated with STK cortical flow. (C-D) The migration and tube formation of LOEC directly correlated with the level of renal net release of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL) -10, respectively.
**Figure S3** The correlation of LOEC function with renal hemodynamics, GFR or blood pressure in patients with EH or ARAS when stratified for statin treatment.

(A-D) In EH patients, the migration of RV LOEC directly correlated with cortical perfusion and GFR in both statin treated and untreated patients. (E-J) Conversely, LOEC proliferation or tube formation inversely correlated with systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP). (K-N) Similarly, in ARAS patients, the migration of RV LOEC directly correlated with cortical perfusion and GFR in both statin treated and untreated patients.